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Abstract

Auricular malformations, which impose a significant social and psychological burden, are currently treated using ear prostheses, synthetic implants or autologous implants derived from rib cartilage. Advances in the field of regenerative medicine and biofabrication provide the possibility to engineer functional cartilage with intricate architectures and complex shapes using patient-derived or donor cells. However, the development of a successful auricular cartilage implant still faces a number of challenges. These challenges include the generation of a functional biochemical matrix, the fabrication of a customized anatomical shape, and maintenance of that shape. Biofabrication technologies may have the potential to overcome these challenges due to their ability to reproducibly deposit multiple materials in complex geometries in a highly controllable manner.

This topical review summarizes this potential of biofabrication technologies for the generation of implants for auricular reconstruction. In particular, it aims to discuss how biofabrication technologies, although still in pre-clinical phase, could overcome the challenges

of generating and maintaining the desired auricular shapes. Finally, remaining bottlenecks and future directions are discussed.

1. Introduction

Auricular malformations, as a result of congenital anomalies, trauma, burns or cancer, impose a significant social and psychological burden on the patient.[1] Improved psychosocial aspects have been documented after auricular reconstruction.[2] Current treatment options include ear prostheses, synthetic implants and auricular reconstruction using skin flaps or autologous rib cartilage. The very first mention of ear reconstruction already dates back to the 6th century BC: the Sushruta Samhita, a Sanskrit text on surgical techniques, describes a cheek flap for earlobe repair.[3] In the 16th and 19th century, various other skin flaps have been used for the partial reconstruction of (traumatic) ear deformities.[4,5] In the early 20th century, techniques have been introduced for complete ear reconstruction. However, these approaches, which used diced and molded rib cartilage, were challenged by progressive resorption.[6-9] In 1937, Gillies even described the repair of more than 30 congenitally malformed external ears (microtia) using ear cartilage from the patient's mother. This approach did, however, not overcome the resorption issues.[10] A major breakthrough in the field of auricular reconstruction came in the form of a carved solid block of autogenous rib cartilage and was introduced by Tanzer in the late 1950s.[11] Modifications of this technique are still regarded the gold standard for auricle reconstruction in patients with microtia.[12-14]

Auricular reconstruction with autologous costal cartilage is, nevertheless, considered an especially challenging procedure in plastic surgery because of the complex three-dimensional (3D) shape of the auricle.[12,15-20] Carving the auricular framework based on the contralateral healthy ear requires significant surgical skill. Differences in surgeon experience, the technique used and tissue handling, together with unpredictable scar tissue formatting, account for marked variability in aesthetic outcome. When creating an auricular implant, the surgeon should emphasize the eminences and depressions of the human auricle (Figure 1), as the overlying skin, which is usually thicker than the skin on the normal ear, will reduce such details.[16] After reconstruction surgery, it also remains a substantial challenge to maintain

the shape of the implant. Costal cartilage, harboring no elastic fibers, lacks the flexibility of a normal ear and can, therefore, appear rigid. In addition, harvesting sufficient amounts of costal cartilage for the hand-carved autogenous implant involves surgery with significant operating time and results in donor site morbidity.[12-15,21,22]

In order to address the increased operating time, donor site morbidity, and intersurgeon shape variability, efforts have been made towards creating prefabricated synthetic auricular implants, including silicone ear frameworks and implants based on nylon and Teflon.[23] Nonetheless, it appeared that these non-degradable synthetic implants were at high risk of extrusion secondary to infection or trauma and were, therefore, deemed unsuitable for reconstruction of the auricle.[12,16,22,24-26] Medpor® a porous high-density polyethylene implant, also evoked concerns of implant exposure, but has regained interest in the past few years when combined with temporoparietal fascial flaps and skin grafts.[27] However, a recent international survey among plastic surgeons showed that the great majority prefers the use of autologous cartilage frameworks over such synthetic implants.[28] Nevertheless, the disadvantages of the current treatment modalities call for a further exploration of alternatives.

Advances in the field of regenerative medicine provide the possibility to engineer functional cartilage using patient-derived or donor cells, overcoming potential rejection of the neo-tissue.[29,30] Such durable cartilage structures can also be generated from auricular cartilage cells and the resulting constructs could be used as auricular implant replacements.[18] Moreover, the convergence of technologies leading to the rapid advancements within the field of biofabrication now allows for the creation of cell-laden implants with intricate architectures and complex shapes.[31] This approach would avoid patient donor-site morbidity and other limitations associated with harvesting costal cartilage and manually sculpting an ear-shaped framework.[18] Based on 3D imaging, implants can be custom-designed to closely match the contralateral ear, resulting in both improved aesthetic and functional outcomes. This review summarizes the potential of biofabrication technologies for the generation of implants for auricular reconstruction. In particular, it aims to discuss how biofabrication technologies, although still in pre-clinical phase, could overcome the challenges of generating and

maintaining the desired auricular shapes. Finally, remaining bottlenecks and future directions are discussed.

2. Challenges in the generation of regenerative auricular implants

Engineering a pre-formed auricle that contains living cells dates back to the 1940s, where diced cartilage grafts and external molds in predetermined ear shapes were used for *in vivo* tissue repair.[32] In the 1970s, after a series of – albeit unsuccessful – experiments, the belief arose that appropriate scaffolds could coax cells into generating new tissue.[33,34] A decade later, the use of synthetic biocompatible, biodegradable polymers as a temporary support structure was suggested[35,36] and the feasibility of generating 3D cartilage constructs was demonstrated by seeding isolated chondrocytes on a fibrous polyglycolic acid (PGA) scaffold.[35] This approach resulted in significant cellular growth and matrix production *in vitro*. Moreover, extended incubation *in vivo* demonstrated histological resemblance to cartilage and maintenance of the 3D shape of the construct.[37] Additional experiments also confirmed that small cell-seeded polymer constructs implanted in nude mice progressively degraded and gradually were – almost entirely – replaced by neo-cartilage. In contrast, control groups with polymer alone or cells alone did not demonstrate new cartilage tissue formation.[35] Although neocartilage was produced within these small constructs, growing tissue-engineered auricular cartilage in a particular complex 3D shape, such as the human auricle, remained a significant challenge.[38] Nearing the end of the century, a major breakthrough was achieved by implanting an engineered ear on the dorsum of nude mice.[39] This new approach involved a mesh of PGA immersed in polylactic acid (PLA), shaped in the form of a human ear, and subsequently seeded with articular chondrocytes. After 12 weeks *in vivo*, implants that were stented externally looked nearly identical to the initial implant. Removal of the skin revealed that a neocartilage framework had actually formed, which was responsible for the – at least temporary – maintenance of shape after removal of the external stent. Implants that were not initially stented externally faced a reduction in size and shape deformation.[39]

These early experiments have paved the way for growing interest in the use of tissue engineering technologies for the generation of viable auricular implants. The ideal engineered

implant should durably match the shape of the contralateral auricle, incorporating autologous chondrocytes or stem cells that have matured into native-like neocartilage tissue, which is strong enough to withstand the contractive forces of the skin and to enable the natural elastic bending of the auricle.[18,40] With time, the scaffold material should slowly degrade while new cartilaginous matrix replaces it, maintaining its original shape.[14,18,41] Next, an auricular implant could even incorporate fatty tissue, perichondrium, or even the covering skin besides the cartilage framework.[42] Taken together, the major challenges faced in the generation of a regenerative auricular implant include the provision a proper environment for tissue growth, remodeling and maturation, the replication and maintenance of the auricular shape, and the generation of constructs that consist of multiple (pre-)tissues.

Microenvironment

It has been suggested that between 100-150 million cartilage cells are required to reconstruct an adult ear[18] and this entire mass of developing cartilage is primarily dependent on diffusion for the supply of oxygen and nutrients. In the native auricle, the cartilage lacks a vascular network and the perichondrium, a thin connective tissue layer surrounding auricular cartilage, is essential in facilitating blood supply to the cartilage surface.[20] Cultured cartilage however, being devoid of a perichondrial layer, completely lacks this vascular supply at the surface in the crucial early stages of development *in vivo*. In particular within larger cartilage constructs, such as for the human auricle, this inevitably leads to profound problems with cell viability and proliferation[20], resulting in inhomogeneous tissue formation[43] and central necrosis.[44]

Besides an adequate supply of nutrients, a stimulatory environment is essential for cell growth, proper differentiation and matrix production. Tissue engineering traditionally involves a mixture of cells, supporting scaffolds, and bioactive cues, *e.g.* growth factors, and the ideal composition of this mixture potentially allows for optimal tissue development.[45,46] Chondrocytes typically thrive best in a soft hydrogel, a highly aqueous cell carrier that allows unimpeded nutrient diffusion and provides a homogenous microenvironment harboring stimulatory components for cellular migration, proliferation and differentiation. Temporarily simulating the natural extracellular matrix of the tissue, hydrogels serve as a guiding support

structure for the deposition of new matrix.[41,47] Just as in naturally developing tissue, cells in engineered constructs – both chondrocytes and stem cells – require guidance of bioactive cues to differentiate towards the (auricular) chondrogenic lineage. Insulin growth factor (IGF), the fibroblast growth factor (FGF) family and the transforming growth factor beta (TGF-β) family appear to be crucial in the development of cartilage tissue.[48]

Creating the shape

The human auricle is a complex 3D shape that includes eminences and depressions formed by the outer helical rim, Y-shaped antihelix, concha bowl, tragus and antitragus.[21] As for reconstructive surgery using costal cartilage, accurately mimicking the shape of the auricle under the skin is also a major challenge for auricular reconstruction using a tissue-engineered implant. Several approaches have been adopted for the generation of engineered cartilage in the shape of the human ear. Many studies have applied hand sculpted and impression molds for the creation of the complex 3D shape of the external ear (Figure 2). [12,13,21,49,50] Molds were, for example, injected with a hydrogel scaffold[50], or polymer sheets seeded with a cell suspension were placed in the mold[12,49] or on a positive cast.[21] Although the initial constructs resembled the shape of the human ear, special attention must be drawn to highlighting the existing eminences and depressions of the auricular framework in order to create a pleasing aesthetic outcome.

Maintaining the shape

Although neocartilage production has been achieved within various scaffold materials and initial satisfactory aesthetic results have also been reported, the majority of studies on bioengineered auricular implants *in vivo* have faced degradation and deformation issues[12,49], exemplifying the need for some form of support during the maturation of the new tissue.[13,39,51] The poor mechanical strength of the construct is partly due to the limited physical properties of the highly aqueous hydrogels. Despite the increased mechanical properties, stiff hydrogels are undesirable as they hinder the cellular processes required for tissue development.[41,52,53] Consequently, it is not surprising that internal support structures, including wire frameworks or

polymer scaffolds, have yielded better results with regards to shape maintenance of newly formed cartilage (Figure 3).[18,40,54-57]

An additional factor that may contribute to the degradation and deformation of engineered constructs is the hampered tissue maturation as a result of limited nutrient supply. In addition, immature and dysmorphic cartilage exhibits significantly less strength than healthy mature cartilage, and is therefore likely to face degradation *in vivo*. This may be of specific importance for larger constructs, *e.g.* for the replacement of an entire auricle, as these are likely to suffer from central cell death and limited proliferation due to nutrient limitation.[20]

Hydrogel-based constructs will exhibit considerably less stiffness than native cartilage tissue.[41] Pre-culture, or "maturation" of constructs before implantation, will improve strength due to matrix deposition. Nevertheless, *in vitro* engineered cartilage constructs do still not have sufficient strength to withstand the contractive forces of the skin.[16,57,58] To date, studies incorporating internal support structures have yielded better outcomes with regards to maintaining dimensions and contours.[40,54-58]

3. Biofabrication-based strategies for auricular reconstruction

The engineering of auricular cartilage constructs thus faces many challenges, including functional biochemical composition, satisfactory anatomy, the creation of a customized shape, and especially the maintenance of that shape. Biofabrication technologies may have the potential to overcome these challenges due to their ability to deposit multiple materials in complex geometries in a highly controllable manner.

Microenvironment

A key issue in tissue engineering is providing the right local cellular environment that promotes cell growth, proper differentiation, and matrix production. Biofabrication technologies can deliver a hybrid construct of the various materials that are required to provide such an environment with high spatial resolution.[41] Hydrogels can function as a building block, as well as a carrier for the cells.[59] These "bio-inks" provide a natural aqueous environment for the cells and have the advantage that they can be processed into a particular shape through biofabrication.[18]

An additional exciting option is using a soluble and printable form of decellularized extracellular matrix (dECM) to create a favorable microenvironment for the encapsulated cells.[60,61] As dECM contains all components of a natural cell environment, it has the potential to greatly enhance cell adhesion, proliferation, organization and maturation.[62] Tissue-specific dECM facilitates specific tissue formation and remodeling, and directs stem cell differentiation and commitment to the determined cell lineage.[60]

Biofabrication technologies can further contribute to the appropriate complexity of the microenvironment for cell growth and differentiation through the delivery of spatially distributed gradients of biochemical cues. For example, various growth factors, including fibroblast growth factor-2 (FGF2), transforming growth factor beta (TGFβ) and insulin growth factor (IGF), could be incorporated and their spatiotemporal release profiles could be tailored towards optimal tissue synthesis and maturation.[63]

Creating the shape

As discussed earlier, accurately mimicking the complex auricular shape is one of the challenges in creating a suitable auricular implant. The medical field already makes use of advanced imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), surface scanning and 3D photography, that are available to aid biofabrication processes through computer-aided design (CAD) and manufacturing (CAM). CAD/CAM technologies can precisely determine the original auricle shape and transform the 3D image data into a manufacturing output file for biofabrication.[64-66] Image-guided design and fabrication has already been used to create meniscus[65] and ear molds for hydrogel-based constructs with fine details.[14] Although such molds allow for gentle shaping of a single cell-seeded material, they do not allow the control of internal material or cellular variations.[67]

Direct deposition of cell-containing hydrogels does allow for the generation of constructs with highly controllable and potentially porous complex configurations that closely resemble native tissue architectures.[67-69] With biofabrication technologies, a high patterning resolution, as well as precise spatial organization of the cellular environment can be achieved using the digital blueprint of a tissue. Different extracellular matrix components, cell types and bioactive molecules, as well as solid biodegradable materials and hydrogels, can be co-deposited into

a specific heterogeneous configuration.[59] The feasibility of such an approach has been demonstrated through the fabrication of a construct consisting of an auricular cartilage framework and fatty tissue earlobe, using co-deposition of two different cell-laden hydrogels within an ear-shaped PCL framework (Figure 5).[42]

The complexity of the shape of the auricle does pose limitations on the building of the implant. One limitation of additive manufacturing techniques is an increase in horizontal cross-sectional area with height, which is the case for the auricle from every angle. The resulting overhangs complicate the printing process. In order to create such a shape without collapsing, either temporary support structures have to be generating during the fabrication process or – alternatively – the construct has to be divided into smaller modules. Alginate[31], Pluronic F127[70] and poly(ethylene) glycol (PEG)[42] are examples of sacrificial support materials that can be applied in biofabrication processes, without having notable detrimental effects on cell viability. In the case of smaller modules, the design of each part should exhibit decreasing horizontal cross-sectional area, so that no sacrificial support layers are required (Figure 6). The parts can later be merged to generate a complete implant. Thus, using these approaches, CAD/CAM and biofabrication technologies have the potential to deliver custom-made implants with high shape-fidelity to the patient.[31,71]

Maintaining the shape

Biofabrication technologies can supply a highly controllable supporting scaffold by incorporating cell-containing hydrogels in a polymer scaffold. Robotic dispensing or inkjet printing principles allow deposition circumstances that can be tailored specifically to the various components of a hybrid construct, co-depositing hydrogels and thermoplastic polymer scaffolds with high spatial resolution (Figure 7).[60,68] Bioprinting permits optimization of the mechanical features of the construct, such as porosity, stiffness and strength, as both composition of the construct and the features of each component can easily be adjusted.[41,69] Native auricular cartilage is a strong yet flexible tissue. Its tensile modulus, a measure of stiffness, has been reported to be approximately 16 MPa.[42] The ultimate tensile strength, the maximum stress a material can withstand, has been reported to be 2.18 MPa for native

auricular cartilage.[72] In contrast, the tensile strength of hydrogels suitable for the encapsulation of cells is generally two to three orders of magnitude lower.[73]

The strength provided by a scaffold is essential to maintain dimensions while the cells produce their extracellular matrix, until the newly formed tissue is strong enough to maintain itself and withstand the contractive forces of the skin. The mechanical strength can be increased by the density of crosslinks (either based on photo-, chemical, or thermal initiation).[41] However, as high polymer crosslinking density and polymer content restrict cell proliferation and migration, the ideal hydrogel scaffold for biofabrication should preferably be composed of a lightly crosslinked bioink that at low concentrations still maintains printing accuracy (Figure 4). The stiffness of a hydrogel-only construct will be inferior to many native tissues, while auricular implants will face challenging contractive skin forces. With biofabrication techniques, biocompatible thermoplastic scaffold structures can be incorporated to increase their structural support. This consequently also allows the use of softer hydrogels as bioinks.[68] The ideal balance of hydrogel/polymer ratio will permit a suitable aqueous cellular microenvironment for cell growth and differentiation, as well as provide adequate strength for shape maintenance. Ultimately, the polymer support network will slowly degrade and be replaced by strong new tissue.[18,41,68]

Maturation and remodeling of the new tissue is an extremely important factor contributing to the end result, as exposing constructs to contractive skin forces early in the maturation process may lead to deformation and degradation of the implant.[14,39,40,57,74] As nutrient limitation causes central cell death, shortening the distance that nutrients have to travel can ensure all areas in a construct have access to sufficient nutrients. One option is to incorporate perfusion channels in the constructs so that during pre-culture nutrient-rich media is allowed access to the more inner parts of the construct. A modular approach (Figure 6), where the implant is made up of separate parts, may be another potential solution. The modules could be matured separately under more controlled conditions than larger engineered constructs can experience, and would be attached to one another once the neo-cartilage is strong enough for implantation under the skin.

Combining multiple tissues in the construct

The human external ear is a complex shape consisting of several tissue types. A normal ear consists of a cartilage framework, coated by perichondrium layer, and then covered by the vascularized skin. Caudal of the cartilage framework is an earlobe consisting of fatty tissue. The auricular implant could consist of just the cartilage framework, and the ear reconstruction will be completed using skin flaps for the creation of the ear lobe. However, biofabrication does provide the opportunity to incorporate the fatty tissue earlobe into the implant[42], or even engineer a complete ear including the covering vascularized perichondrium and skin.

As pointed out earlier, one study used 3D printing technology to create a composite tissue in the shape of the human auricle, incorporating both chondrocytes and adipocytes for the regeneration of the cartilage framework and the fatty tissue earlobe, respectively.[42] The cells were printed separately in their respective locations within an ear-shaped polymer framework. Although the above-mentioned study demonstrated that co-fabrication of multiple tissues within one construct is technically feasible, the control and regulation of the simultaneous generation of multiple types of tissue in a single construct is still a challenge and further *in vitro* and *in vivo* analysis is required.[42]

4. Future perspectives

This review addresses the various challenges in engineering a viable implant for auricular reconstruction. A first challenge for auricular implants is the design of the intricate shape and the subsequent maintenance of that shape. Biofabrication technologies are able to create complex 3D constructs with a highly detailed internal and external architecture. Auricular reconstruction is an aesthetic practice and, therefore, requires a personalized approach. Ultimately, the design of a restorative auricular implant should closely match the shape of the contralateral ear in order to achieve the best results. CAD/CAM technology has the potential to provide these patient-specific shapes for the design of the implant and can thus play an important role in personalized medicine approaches.

Biofabrication technologies also have the capacity to incorporate various materials into hybrid structures, including live cells, natural matrix components and reinforcing polymer fibers. The addition of bioactive cues, such as growth factors, or decellularized extracellular matrix to the cellular microenvironment can enhance growth and differentiation. The

biofabrication of auricular cartilage implants is still in its infancy, and additional optimization of construct composition and structure is still required until conditions for routine clinical application are attained. As the insertion of artificial materials could elicit any degree of foreign body response or rejection by the immune system – causing inflammation and possible deformation of the construct or extrusion of the materials through the skin – the immunologic response to, as well as the carcinogenic potential of such materials, should be carefully evaluated before translation to the clinic.

An additional important issue in tissue engineering is the improvement of the accessibility of nutrients within constructs and the subsequent maturation of the neo-tissue. Although aberrant from normal cartilage tissue where the surrounding vascularized tissues are responsible for nutrient supply to the mature cartilage, a possible solution is the incorporation of a (temporary) engineered perfusion network within the construct. An alternative approach is the design of a modular construct, in which parts of the complete implant are matured separately. The design should result in modules that are accessible for nutrients by diffusion to ensure proper tissue maturation. Such auricular modules could then be matured separately in an *in vitro* and/or *in vivo* bioreactor.[18] Nevertheless, subsequent integration of the modules still needs to be addressed, as this has been shown to be dependent of the degree of maturation of the neocartilage tissue.[75,76]

Despite a maturation phase, the developing tissue will initially exhibit only limited mechanical strength. For auricular implants, however, initial mechanical integrity is of utmost importance as the contractive forces of the covering skin may cause degradation and deformation of the construct. To overcome this issue, cell-laden hydrogels can be reinforced with a polymer fiber network for increased mechanical strength. Such hybrid constructs exhibit increased mechanical strength as demonstrated by a higher Young's modulus and ultimate tensile strength.[77] Fiber reinforcement of hydrogels can be applied in a layer-by-layer fashion through multi-head robotic dispensing, inkjet printing, or organized microfiber deposition through electrospinning.[68,77,78] In order to select appropriate reinforcing polymers, extensive evaluation of the printability, cytocompatibility, degradation and (temporal) mechanical strength of candidate materials is required.

The next step towards more complex tissue engineering in biofabrication is the co-deposition of multiple pre-tissue types within a single construct.[79] Although a biofabricated ear could consist of solely the auricular implant, it may also incorporate the fatty tissue earlobe[42], or even the covering perichondrium and skin. Engineering the auricle with its multiple tissue types and complex shape can be a step towards increased complexity in tissue engineering. Furthermore, the successful integration of functional nanoelectrical components within the biologically active engineered tissue[80] (Figure 8) further underscores the versatility and potential of biofabrication technologies towards creating more complex and functional structures, tissue parts or eventually whole organs.

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FIGURE LEGENDS

Figure 1: Anatomy of the human auricle, emphasizing the eminences and depressions of the three-dimensional shape.

Figure 2: Examples of tissue engineered ear shapes: PGA/PLLA mesh using a negative mold and seeded with chondrocytes (A). Reprinted with permission from Shieh *et al.* (2004).[12] Gold negative mold filled with chondrocytes mixed with various biodegradable polymers (B). Reprinted with permission from Kamil *et al*. (2004).[50] Silicone mold filled with PLLA/PGLA polymer scaffold and seeded with chondrocytes (C). Reprinted with permission from Haisch *et al*. (2002).[54]

Figure 3: Tissue engineered ear shape using a metal wire framework as an internal support structure to maintain dimensions. Reprinted with permission from Cervantes *et al*. (2013).[40]

Figure 4: The ideal biofabrication window allows high printing accuracy, sufficient mechanical stability and optimal cell conditions. Reprinted with permission from Malda *et al.* (2013).[41]

Figure 5: The co-deposition of multiple hydrogels within the auricular three-dimensional (3D) shape. Reprinted with permission from Lee *et al.* (2014).[42] Reproduced by permission of IOP Publishing.

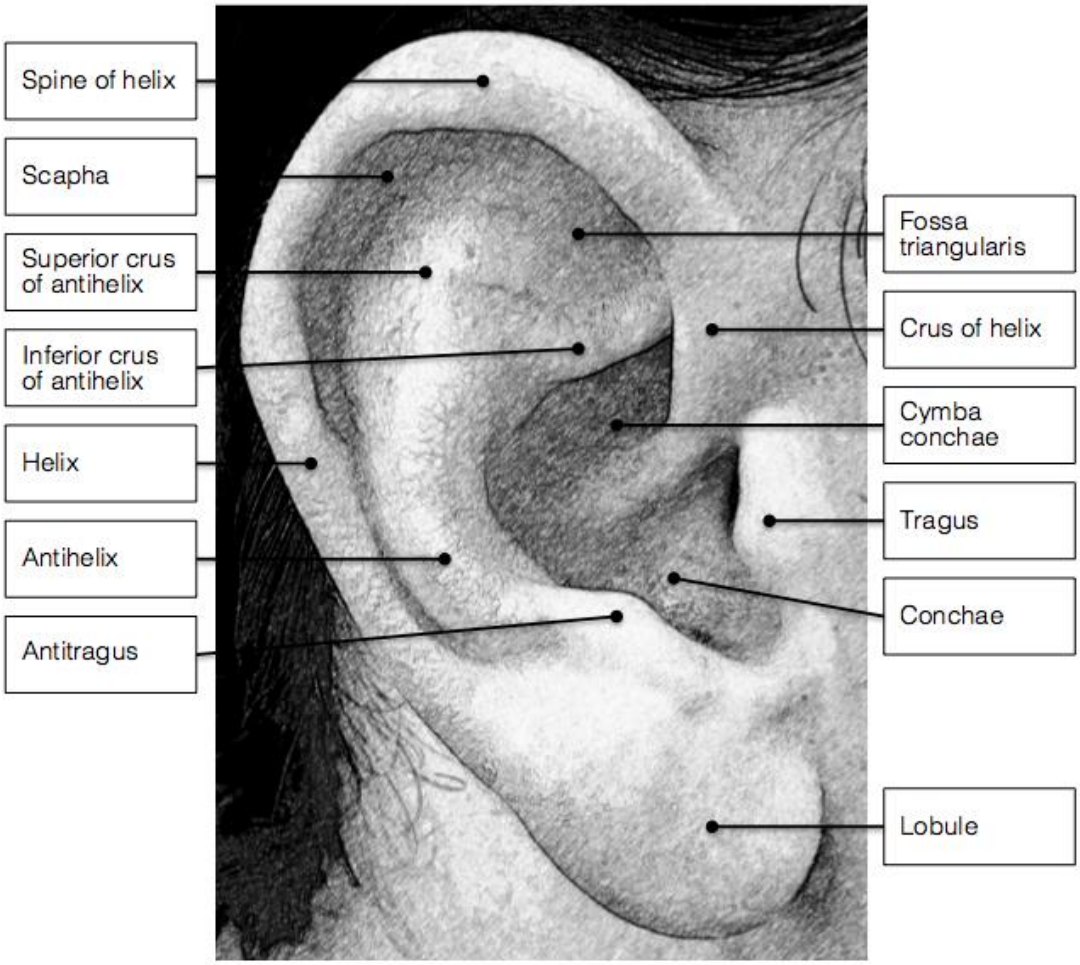
Figure 6: Modular approach where each module exhibits decreasing cross-sectional diameter (A) and allowance of adequate oxygen gradient. Assembled modular construct printed in PCL (B) that displays satisfactory aesthetic appearance under rubber 'skin' (C).

Figure 7: Bioprinting allows for the co-deposition of hydrogels and reinforcing polymer scaffolds with high spatial resolution. Reprinted with permission and adapted from Schuurman *et al*. 2011.[68] Reproduced by permission of IOP Publishing.

Figure 8: The bionic ear: the integration of functional nanoelectrical components within the tissue engineered auricle. Reprinted with permission from Mannoor *et al.* (2013).[80]

FIGURES

Figure 1:



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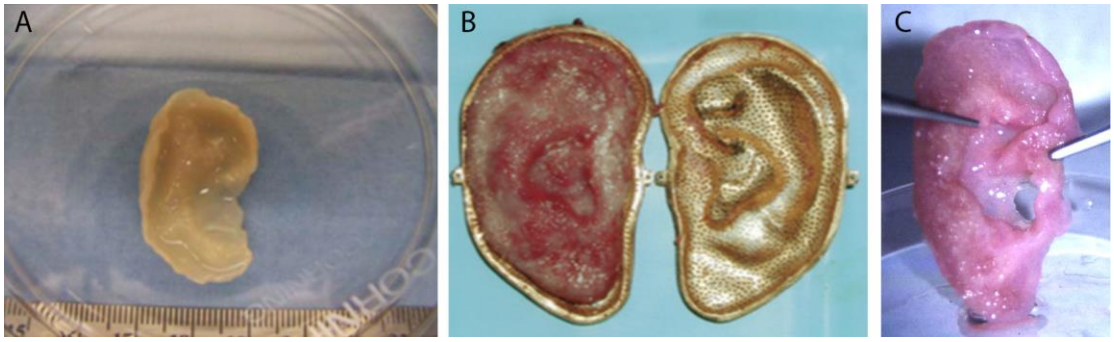

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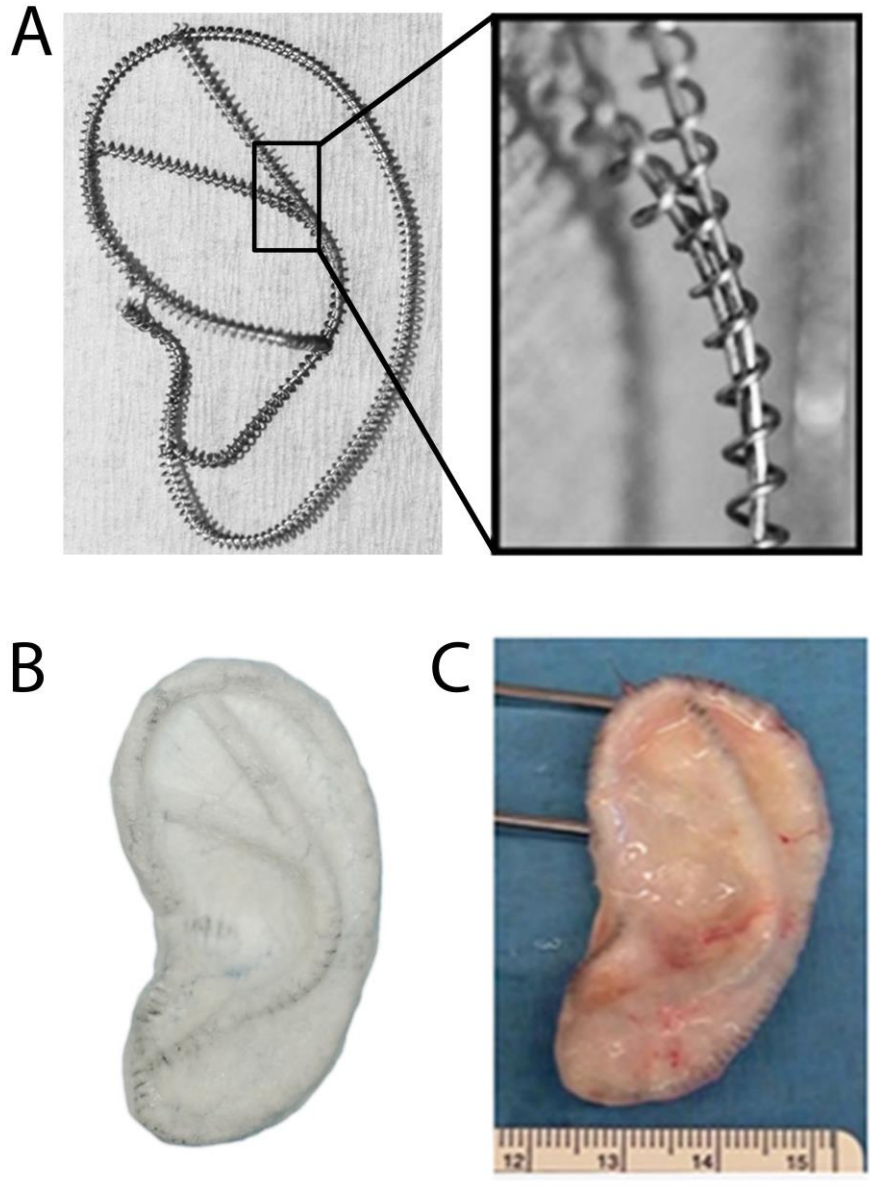

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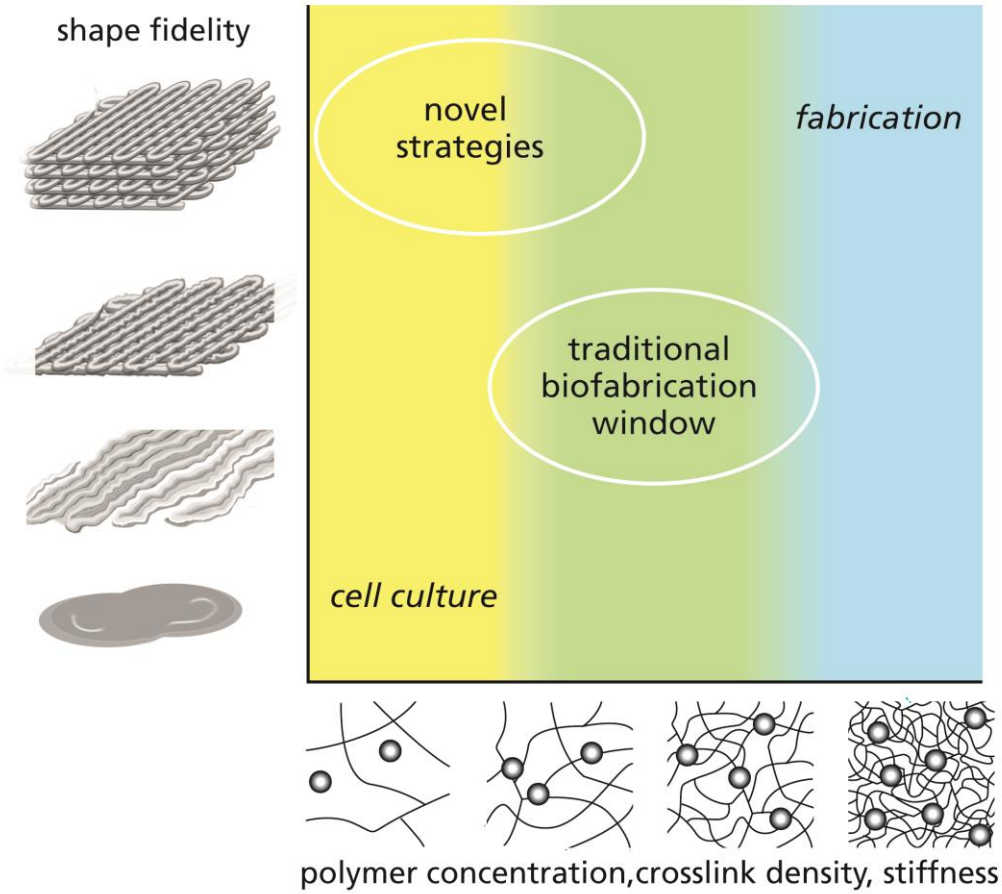
shape fidelity
novel
strategies
fabrication
traditional
biofabrication
window
cell culture
polymer concentration,crosslink density, stiffness

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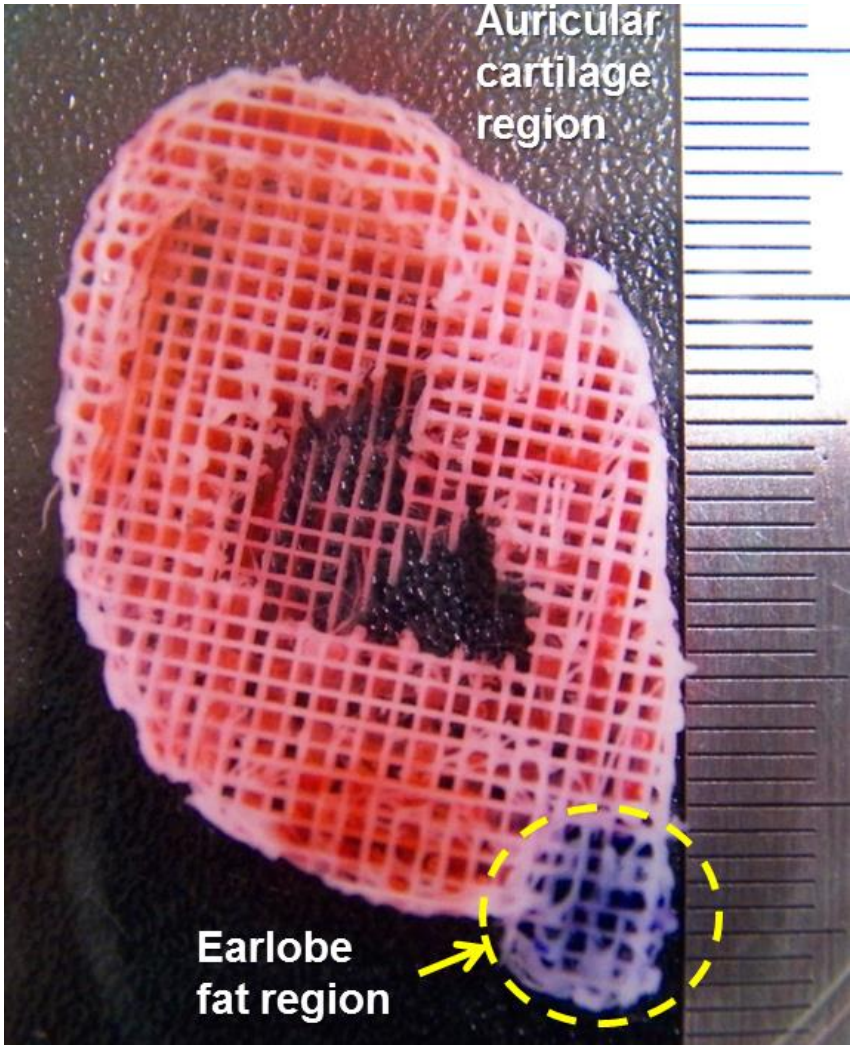

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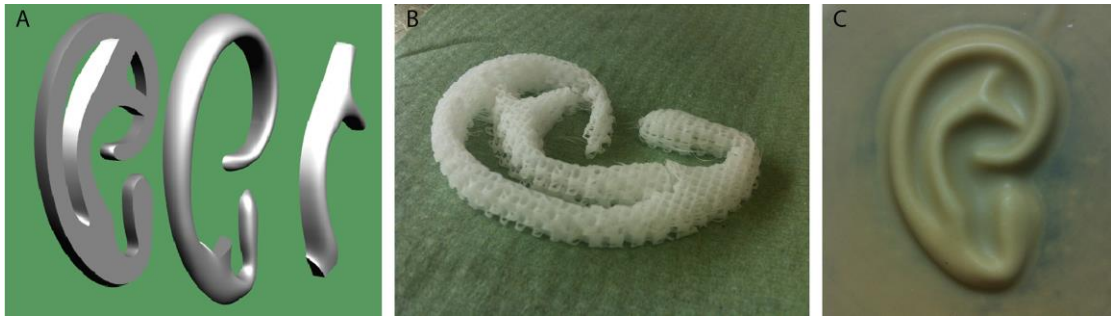

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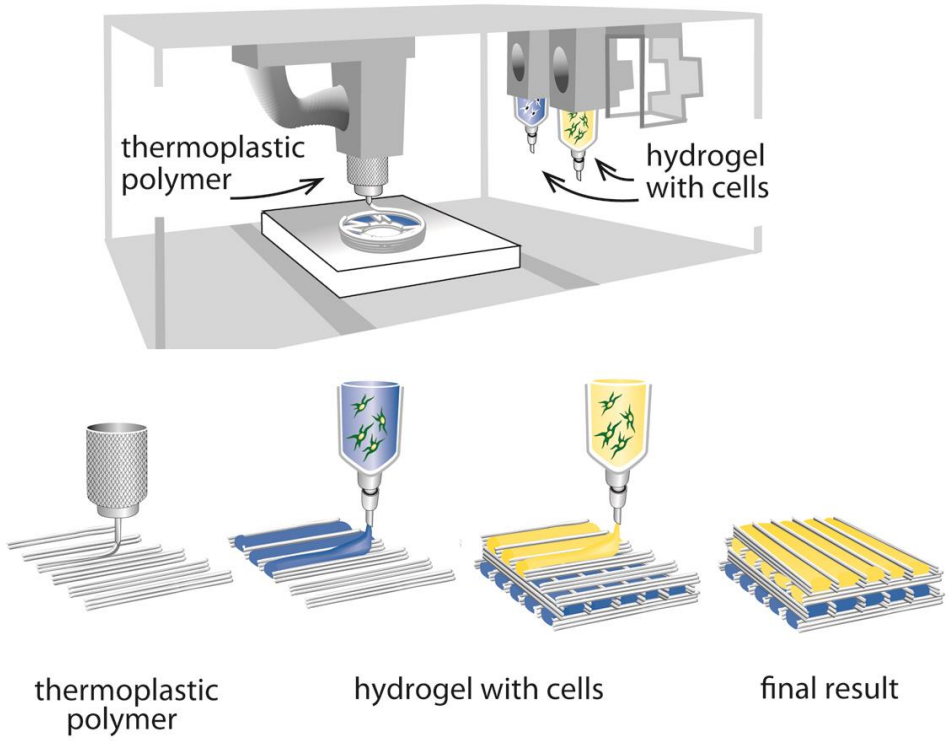
thermoplastic
polymer
hydrogel
with cells
thermoplastic
polymer
hydrogel with cells
final result

Figure 8: